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TITLE: BIOSENSORS BASED ON DIRECTED
                      ASSEMBLY OF PARTICLES

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BIOSENSORS BASED ON DIRECTED ASSEMBLY OF PARTICLES

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

BACKGROUND

Besides proteins, nucleic acids have also been found to have catalytic activities in recent years. The catalytic active nucleic acids can be catalytic DNA/RNA, also known as DNAzymes/RNAzymes, deoxyribozymes/ribozymes, and DNA enzymes/RNA enzymes. Catalytic active nucleic acids can also contain modified nucleic acids. The catalytic activities of nucleic acid-based enzymes always depend on the presence of certain cofactors, for example, metal ions. Therefore, nucleic acid enzyme-based biosensors for these cofactors (e.g. biosensors for metal ions) can be designed based on the activities of the corresponding nucleic acid enzymes. On the other hand, nucleic acids may be selected to bind to a wide range of analytes with high affinity and specificities. These binding nucleic acids are known as aptamers.

Aptamers are nucleic acids (such as DNA or RNA) that recognize targets with high affinity and specificity (Ellington and Szostak 1990, Jayasena 1999). Aptazymes (also called allosteric DNA/RNAzymes or allosteric (deoxy)ribozymes) are DNA/RNAzymes regulated by an effector (the target molecule). They typically contain an aptamer domain that recognizes an effector and a catalytic domain (Hesselberth et al. 2000, Soukup and Breaker 2000, Tang and Breaker 1997). The effector can either decrease or increase the catalytic activity of the aptazyme through specific interactions between the aptamer domain and the catalytic domain. Therefore, the activity of the

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aptazyme can be used to monitor the presence and quantity of the effector. This strategy has been used to select and design aptazyme sensors for diagnostic and sensing purposes (Breaker 2002, Robertson and Ellington 1999, Seetharaman et al. 2001). DNAzymes and DNA aptazymes are the most

5   attractive candidate for sensor development because DNA is much less expensive to synthesize and more stable than RNA. In addition, general strategies to design DNA aptazymes, by introducing aptamer motifs close to the catalytic core of DNAzymes, are available (Wang et al. 2002). High cleavage activity requires the presence of effector molecules that upon binding to the

10   aptamer motif, can allosterically modulate the activity of the catalytic core part of the aptazyme.

In vitro selection methods can be used to obtain aptamers for a wide range of target molecules with exceptionally high affinity, having dissociation constants as high as in the picomolar range (Brody and Gold 2000, Jayasena

15   1999, Wilson and Szostak 1999). For example, aptamers have been developed to recognize metal ions such as Zn(II) (Ciesiolka et al. 1995) and Ni(II) (Hofmann et al. 1997); nucleotides such as adenosine triphosphate (ATP) (Huizenga and Szostak 1995); and guanine (Kiga et al. 1998); co-factors such as NAD (Kiga et al. 1998) and flavin (Lauhon and Szostak 1995); antibiotics such as viomycin

20   (Wallis et al. 1997) and streptomycin (Wallace and Schroeder 1998); proteins such as HIV reverse transcriptase (Chaloin et al. 2002) and hepatitis C virus RNA-dependent RNA polymerase (Biroccio et al. 2002); toxins such as cholera whole toxin and staphylococcal enterotoxin B (Bruno and Kiel 2002); and bacterial spores such as the anthrax (Bruno and Kiel 1999). Compared to

25   antibodies, DNA/RNA based aptamers are easier to obtain and less expensive to produce because they are obtained in vitro in short time periods (days vs. months) and with limited cost. In addition, DNA/RNA aptamers can be denatured and renatured many times without losing their biorecognition ability. These unique properties make aptamers an ideal platform for designing highly sensitive

30   and selective biosensors (Hesselberth et al. 2000).

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To assay DNA/RNAzyme or aptazyme activity, detectable labels are used. However, many of these suffer from significant drawbacks. Radioisotopes incur safety and disposal concerns, whereas fluorophores may undergo photo-bleaching and may also inhibit the biological activity trying to be assayed; organic

5   dyes, such as that used in a cocaine-detecting aptamer system (Stojanovic and Landry, 2002) require high concentrations for visual detection and are matched to a specific aptamer only after costly trial and error.

Metallic particles overcome all of these difficulties. They can be used in small (nanomolar) amounts as detection agents with aptamers without any of the

10 disadvantages of organic dyes. In sensors based on aptamers using metallic particles for color detection, the cleavage of a nucleic acid substrate by the aptazyme (upon binding of an effector) may be detected by color changes.

Typically, a DNA/RNAzyme- or aptazyme-based sensor has three parts:

(1)   a nucleic acid enzyme (in the following description, nucleic acid

15 enzymes will be referred to DNA/RNAzymes and aptazymes) and a co-factor, such as a metal ion that catalyzes substrate cleavage;

(2)   a nucleic acid substrate for the nucleic acid enzyme, wherein interior portions of the substrate sequence is complementary to portions of the enzyme sequence; and

20 (3)   particles attached to polynucleotides that are complementary to the 3'- and 5'- termini of the substrate.

To detect the target cofactor or effector, the complementary portions of the polynucleotides (the polynucleotides attached to the particles complementary to the 3'- and 5'-termini of the substrate strand, and the 5'- and 3'-termini of the

25 nucleic acid enzyme complementary to interior substrate strand sequences) are annealed in the presence of a sample suspected of containing the targeted cofactor or effector. If the cofactor or effector is absent, the aptazyme is either inactive or shows substantially reduced activity, resulting in no or little substrate cleavage and thus aggregation of the particles. If the cofactor or effector is

30 present, the enzyme becomes active and cleaves the substrate, preventing

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aggregate formation because the link between the particles is broken by enzymatic cleavage. Table 1 exemplifies such a system.

Cleavage	Aggregation	Color*
YES (the target effector is present)	DISPERSED	RED
NO	NON-DISPERSED	BLUE

* When gold particles are used for detection.

5   In the case of gold particles, the aggregated state displays a blue color, while the dispersed state (or the non-aggregate state) is red in color. The presence of the target analyte as a cofactor or effector can be detected based on the appearance of the color of the sensor system.

Since the degree of cleavage is reflected in the degree of color change, 10   the target cofactor or effector concentration can be quantified. For example, simple spectrometry may be used for sensitive detection. Not only can color change be used for detection and quantifying, other results of the cleavage may be employed, such as precipitation. By replacing the aptamer domain with aptamers recognizing pre-selected effectors, colorimetric sensors for any desired 15   effector can be easily made and used.

Based on previous work, a colorimetric biosensor for Pb(II) based on the DNAzyme-directed assembled of gold nanoparticles and a colorimetric biosensor for adenosine based on the aptazyme-directed assembled of gold nanoparticles have been designed (see, for example, U.S. application Ser. Nos. 09/605,558; 20   10/144,094; 10/144,679; and 10/384,497). Though highly sensitive and selective, this type of analytical sensor requires heating to above 50 °C for several minutes and cooling slowly to room temperature in 2 hours for detection.

SUMMARY

25   In a first aspect, the invention is a sensor system for detecting an effector or cofactor, comprising (a) a nucleic acid enzyme, comprising a cofactor binding

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site and optionally an effector binding site; (b) substrates for the nucleic acid enzyme, comprising first polynucleotides; (c) a first set of particles comprising second polynucleotides, where the polynucleotides are attached to the particles at the 3' terminus; and (d) a second set of particles comprising third

5   polynucleotides, where the polynucleotides are attached to the particles at the 5' terminus. The first polynucleotides comprise or are at least partially complementary to the second polynucleotides, and the first polynucleotides comprise or are at least partially complementary to the third polynucleotides.

In a second aspect, the present invention is sensor system for detecting

10   an effector or cofactor, comprising (a) a nucleic acid enzyme, comprising a cofactor binding site and optionally an effector binding site; (b) substrates for the nucleic acid enzyme, comprising first polynucleotides; (c) a first set of particles comprising second polynucleotides; and (d) a second set of particles comprising third polynucleotides. The first polynucleotides comprise or are at least partially

15   complementary to the second polynucleotides, and the first polynucleotides comprise or are at least partially complementary to the third polynucleotides. The second set of particles have a diameter of at least 20 nm.

In a third aspect, the present invention is a method of detecting an effector or cofactor in a sample, comprising mixing together at least (a) a

20   nucleic acid enzyme, comprising a cofactor binding site and optionally an effector binding site; (b) substrates for the nucleic acid enzyme, comprising first polynucleotides; (c) a first set of particles comprising second polynucleotides; (d) a second set of particles comprising third polynucleotides; and (e) the sample; to form a mixture. The first polynucleotides comprise or

25   are at least partially complementary to the second polynucleotides, and the first polynucleotides comprise or are at least partially complementary to the third polynucleotides. The mixture produces a color change indicating the presence of the effect or cofactor in the sample within 10 minutes of forming the mixture, without heating.

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DESCRIPTION OF THE FIGURES

Figure 1. (A) Secondary structure of the "8-17" DNAzyme system that consists of an enzyme strand (17E) and a substrate strand (17DS). (B) Cleavage of 17DS by 17E in the presence of Pb(II). (C) Schematics of DNAzyme-directed assembly of gold particles and their application as biosensors for metal ions such as Pb(II).

Figure 2. (A) The primary and the proposed secondary structure of the adenosine aptazyme built on the "8-17" DNAzyme platform. (B) Schematic representation of the colorimetric detection of adenosine.

Figure 3. The two alignments of DNA linked gold particles. (A) "head-to-tail" aligned particles, where only one kind of thiol-modified DNA is needed. (B) "tail-to-tail" aligned particles

Figure 4. The effect of particle alignment and particle size on the rate of color change.

Figure 5. A Pb^{2+} colorimetric biosensor where the diameter of particles is ~35 nm and the particles are aligned in the "tail-to-tail" manner.

Figure 6. A new Pb^{2+} sensor system. (A). The extinction spectra of 35 nm gold particles at different times. (B) Effect of substrate concentration on the rate of aggregation. (C) Effect of NaCl concentration on the rate of aggregation. (D) Effect of 17E concentration on the rate of aggregation. (E) Aggregation of particles in different pH buffers. (f) Extinction ratio after 10 minutes of aggregation.

Figure 7. Sensitivity and selectivity of a new Pb^{2+} sensor. (A) Kinetics of gold particle aggregation with different Pb^{2+} concentrations. (B) The extinction ratio at 10 minutes after the initiation.

Figure 8. A new design of an aptazyme-based adenosine sensor. Reaction A: formation of blue aggregates in the absence of adenosine. Reaction B: the substrate is cleaved in the presence of adenosine. Reaction C: the cleaved substrate cannot assemble gold particles, yielding red-colored,

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separated particles.

Figure 9. (A) Substrate concentration-dependent aggregation of particles. (B) Kinetics of the cleavage of the substrate by the aptazyme. (C) Sensitivity and selectivity of an aptazyme-based sensor for adenosine.

DETAILED DESCRIPTION

The present invention avoids the heating required for previous DNAzyme- and aptazyme-metal particle sensor systems, allowing these sensors to be used not only in laboratories, but by consumers in their homes, or by technicians in the field. The invention uses the following discoveries that influence the performance of particle-based colorimetric biosensors: (1) altering the alignment of the particles from head-to-tail to tail-to-tail; (2) using larger particles; and (3) controlling ionic strength, aptamer concentration and pH.

The aggregation of particles is influenced by their alignment with respect to each other. Particles may be aligned in two ways, "head-to-tail" or "tail-to-tail" (Figure 3). In an earlier DNAzyme-based Pb^{2+} sensor, for example, the particles are aligned in the "head-to-tail" fashion. If the particles are aligned in the "head-to-tail" manner, only one kind of thiol-modified DNA is needed to attach the DNA to the particle. In this configuration, it is difficult for the particles to aggregate, possibly due to steric effects. Heating and cooling is thus necessary to promote the assembly of particles. However, it has now been discovered that when a "tail-to-tail" alignment is used, the particles can aggregate at ambient temperature (Figure 5). For "tail-to-tail" aligned particles, both 3' - and 5' -thiol-modified polynuclotides are needed.

The color of particle aggregates is mainly governed by the size of the aggregates. Thus, the rate of aggregation being equal, the rate of color change increases using larger particles. As illustrated in the examples below, when a mixture of 13 and 35 nm diameter particles is used, the rate of color change is faster than with particles of 13 nm diameter only. The rate of color change further increases if only particles of 35 nm diameter are used.

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The ionic strength of the solution influences the performance of the system as well. Higher salt concentration favors aggregation, likely due to the salt reducing the electrostatic repulsion between the negatively charged polynucleotide strands. Though only the substrate strand is needed to link two particles, in the absence of the enzyme strand the substrate is too "floppy" to assemble particles. Thus the concentration of the enzyme is an important parameter.

pH also influences aggregation. The influence of pH is likely due to protonation of the bases of the polynucleotide, which affects base pairing and thus the rate of aggregation.

Definitions

A "co-factor" is an ion or molecule involved in the catalytic process of nucleic acid enzyme-catalyzed reactions and is required for catalytic activity.

An "effector" is a molecule that, when bound to an enzyme having an effector binding site, can enhance or inhibit enzyme catalysis. An "effector binding site" may be "specific," that is, binding only one effector molecule in the presence of other effector molecules. An example of effector binding site specificity is when only an adenosine molecule binds in the presence of many other similar molecules, such as cytidine, gaunosine and uridine. Alternatively, an effector binding site may be "partially" specific (binding only a class of molecules), or "non-specific" (having molecular promiscuity). Examples of effectors include environmental pollutants, such as nitrogen fertilizers, pesticides, dioxin, phenols, or 2,4-dichlorophenoxyacetic acid; heavy metal ions, such as Pb(II), Hg(II), As(III), UO_2(II), Fe(III), Zn(II), Cu(II), or Co(II); biological molecules, such as glucose, insulin, hCG-hormone, HIV or HIV proteins; chemical and biological terrorism agents, such as anthrax, small pox, or nerve gases; explosives, such as TNT or DNT; drugs, such as cocaine or antibiotics.

A "nucleic acid enzyme" is an enzyme that principally contains nucleic acids, such as ribozymes (RNAzymes), deoxyribozymes (DNAzymes), and

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aptazymes. Nucleic acids may be natural, unnatural or modified nucleic acids. Peptide nucleic acids (PNAs) are also included. A nucleic acid enzyme requires a "co-factor" for efficient substrate cleavage and/or specific effector binding. Common co-factors include Mg(II), Ca(II), Zn(II), Mn(II), Co(II) and Pb(II).

5   "Polynucleotide" refers to a nucleic acid sequence having at least two or more nucleotides. Polynucleotides may contain naturally-occurring nucleotides and synthetic nucleotides. PNA molecules are also embraced by this term.

          "Sensitivity" refers to the smallest increase of a cofactor or effector concentration that can be detected by the sensor.

10   "Detection limit" refers to the limits of detection of an analytical device. In the context of the DNAzyme- and aptazyme-based sensors of the present invention, detection limit refers to the lowest concentration of a cofactor or effector that the sensor can differentiate from the background.

          "Base-pairing" refers to the ability of a polynucleotide to form at least one hydrogen bond with a nucleotide under low stringency conditions. The nucleotide may be part of a second polynucleotide or to a nucleotide found within the first polynucleotide. A polynucleotide is partially complementary to a second polynucleotide when the first polynucleotide is capable of forming at least one hydrogen bond with the second polynucleotide. To be partially complementary, a polynucleotide may have regions wherein base pairs may not form surrounded by those regions that do, forming loops, stem-loops, and other secondary structures.

          "Aptamer" refers a polynucleotide which contains an effector binding cite. An "effector binding site" may be "specific," that is, binding only one effector molecule in the presence of other effector molecules. An example of effector binding site specificity is when only an adenosine molecule binds in the presence of many other similar molecules, such as cytidine, gaunosine and uridine. Alternatively, an effector binding site may be "partially" specific (binding only a class of molecules), or "non-specific" (having molecular promiscuity).

30   "Aptazyme" refers to a nucleic acid enzyme that includes an aptamer

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region which binds an effector. The binding of the effector can enhance or inhibit catalysis.

A nucleic acid enzyme having an effector (or effectors) binding site

5   A number of nucleic acid enzymes have been discovered or developed, having diverse catalytic activities (Tables 1 and 2). For catalytic function, the enzymes usually depend on one or more co-factors. *In vitro* selection may be used to "enhance" selectivity and sensitivity for a particular ion. Nucleic acid enzymes that catalyze molecular association (ligation, phosphorylation, and

10   amide bond formation) or dissociation (cleavage or transfer) are particularly useful in the present invention.

        A nucleic acid enzyme that catalyzes the cleavage of a nucleic acid in the presence of an effector is used. The nucleic acid enzyme may be RNA (ribozyme), DNA (deoxyribozyme), a DNA/RNA hybrid enzyme, or a peptide

15   nucleic acid (PNA) enzyme. PNAs comprise a polyamide backbone and nucleoside bases (available from, *e.g.,* Biosearch, Inc. (Bedford, MA)). Ribozymes that may be used include group I and group II introns, the RNA component of the bacterial ribonuclease P, hammerhead, hairpin, hepatitis delta virus and *Neurospora* VS ribozymes. Also included are *in vitro* selected

20   ribozymes, such as those previously isolated (Tang and Breaker 2000). Ribozymes tend to be less stable than deoxyribozymes; thus deoxyribozymes are preferred. Deoxyribozymes with extended chemical functionality are also desirable (Santoro *et al.*, 2000).

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Table 1 Reactions catalyzed by ribozymes that were isolated from *in vitro* selection experiments.

Reaction	k_{cat} (min^{-1})	K_m (µM)	k_{cat}/k_{uncat} [a]	Reference
Phosphoester centers				
Cleavage	0.1	0.03	10^5	(Vaish et al. 1998)
Transfer	0.3	0.02	10^{13}	(Tsang and Joyce 1996)
Ligation	100	9	10^9	(Ekland et al. 1995)
Phosphorylation	0.3	40	$>10^5$	(Lorsch and Szostak 1994)
Mononucleotide polymerization	0.3	5000	$>10^7$	(Ekland and Bartel 1996)
Carbon centers				
Aminoacylation	1	9000	10^6	(Illangasekare and Yarus 1997)
Aminoacyl ester hydrolysis	0.02	0.5	10	(Piccirilli et al. 1992)
Aminoacyl transfer	0.2	0.05	10^3	(Lohse and Szostak 1996)
N-alkylation	0.6	1000	10^7	(Wilson and Szostak 1995)
S-alkylation	4×10^{-3}	370	10^3	(Wecker et al. 1996)
Amide bond cleavage	1×10^{-5}		10^2	(Dai et al. 1995)
Amide bond formation	0.04	2	10^5	(Wiegand et al. 1997)
Peptide bond formation	0.05	200	10^6	(Zhang and Cech 1997)
Diels-Alder cycloaddition	>0.1	>500	10^3	(Tarasow et al. 1997)
Others				
Biphenyl isomerization	3×10^{-5}	500	10^2	(Prudent et al. 1994)
Porphyrin metallation	0.9	10	10^3	(Conn et al. 1996)

[a] Reactions catalyzed by ribozymes that were isolated from *in vitro* selection experiments.
k_{cat}/k_{uncat} is the rate enhancement over uncatalyzed reaction.

Table 2   Deoxyribozymes isolated through *in vitro* selection.

Reaction	C factor	$k_{max}(\text{min}^{-1})^b$	k_{cat}/k_{uncat}	Reference
RNA transesterification	Pb^{2+}	1	10^5	(Breaker and Joyce 1994)
	Mg^{2+}	0.01	10^5	(Breaker et al. 1995)
	Ca^{2+}	0.08	10^5	(Faulhammer and Famulok 1997)
	Mg^{2+}	10	$>10^5$	(Santoro and Joyce 1997)
	None	0.01	10^8	(Geyer and Sen 1997)
	L-histidine	0.2	10^6	(Roth and Breaker 1998)
	Zn^{2+}	~40	$>10^5$	(Li et al. 2000)
DNA cleavage	Cu^{2+}	0.2	$>10^6$	(Carmi et al. 1996)
DNA ligation	Cu^{2+} or Zn^{2+}	0.07	10^5	(Cuenoud and Szostak 1995)
DNA phosphorylation	Ca^{2+}	0.01	10^9	(Li and Breaker 1999)
5',5'-pyrophosphate formation	Cu^{2+}	5×10^0	$>10^{10}$	(Li et al. 2000)
Porphyrin metalation	None	1.3	10^3	(Li and Sen 1996)

$^b k_{max}$ is the maximal rate constant obtained under optimized conditions.

Methods of producing ribozymes and deoxyribozymes include chemical oligonucleotide synthesis, polymerase chain reaction (PCR), DNA cloning and replication. Preferably the nucleic acid enzymes are DNA/RNA hybrids and PNAs. Nucleotides containing modified bases, phosphates, or sugars may also

5   be used; in some instances, these modified nucleotides may be advantageous for stability or confer effector specificity. Examples of modified bases include inosine, nebularine, 2-aminopurine riboside, N^7-deazaadenosine, and O^6-methylguanosine (Earnshaw and Gait 1998). Modified sugars and phosphates include 2'-deoxynucleoside, abasic, propyl, phosphorothioate, and 2'-O-allyl

10   nucleoside (Earnshaw and Gait 1998).

A nucleic acid enzyme that cleaves a nucleic acid strand separate from the strand comprising the enzyme is a *trans*-acting enzyme. Using *trans*-acting enzymes allows for multiple rounds of substrate cleavages, since the enzymatic product is removed. An example of such a nucleic acid enzyme is 17E (SEQ ID

15   NO:1); the corresponding substrate is 17DS (SEQ ID NO:2; r denotes a single ribonucleotide); both are presented in Table 3A and illustrated in Figure 1. The secondary structure of the "8-17" DNAzyme system, including an enzyme strand (17E) and a substrate strand (17DS), is illustrated in Figure 1a. The cleavage site is indicated by an arrow. Except for a ribonucleoside adenosine

20   at the cleavage site (rA), all other nucleosides are deoxyribonucleosides. In the presence of Pb(II), the enzyme strand cleaves the substrate strand (Figure 1b). Thus, as illustrated in Figure 1c, the enzyme strand and the substrate strand may be used in the DNAzyme-directed assembly of gold particles and in its application as a biosensor for metal ions such as Pb(II). In

25   this system, the 17DS has been extended on both the 3' and 5' ends for 12 bases, which are complementary to the 12-mer DNA attached to the 13 nm gold particles (DNA$_{Au}$). Other examples are also given in Table 3B.

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Table 3A   DNA enzymes and substrates

Molecule	SEQ ID NO:	Sequence	# of nucleotides
Enzyme (17E)	1	5'-catctcttct ccgagccggt cgaaatagtg agt-3'	33
Substrate for 17E (17DS)[c]	2	5'-actcactatr ggaagagatg-3'	20
Enzyme: JLYL1 "8-17" half	5	5'-tctcttctcc gagccggtcg aaatattgga ggaagctc-3'	38
ATP half	6	5'-gagctggagg aaaaagtgag tc-3'	22
Sustrate for JLYL1	4	5'-gactcactat rggaagaga-3'	19
Enzyme: JLYL2 "8-17" half	8	5'-tctcttct ccgagccggt cgaaatattg gaggaagctc-3'	38
ATP half	9	5'-gagctggagg aaaaagtgag tc-3'	22
Substrate for JLYL2	7	5'-actcatctgt gagactcact atrggaagag atgtcaactc gtg-3'	43

[c]"r" denotes a single ribonucleotide

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Table 3B   RNA/DNA based aptamers and RNA/DNAzymes

RNA/DNA based aptamers and their targets[1-4]		RNA/DNAzymes[5],[6-10]
Organic dyes[11,12]	Xanthene[59]	8-17 DNAzyme[13-15]
Theophyllin[16]	Kanamycin A[60]	10-23 DNAzymes[13,17]
Dopamine[18]	Lividomycin[60]	Hammerhead[19,20]
Hoechst 33258[21]	Tobramycin[61]	Hairpin[19,22]
Sulforhodamine B[23,24]	Neomycin B[62,63]	Leadzyme[25]
Cellobiose[26]	Viomycin[64]	Hepatitis Delta Virus[27,28]
D-tryptophan[29]	Chloramphenicol[65]	Group I Intron[30,31]
L-arginine[32-37]	Streptomycin[66]	Spliceosome[38]
L-citrullin[32,36]	HIV-1 Rev peptide[67,68]	Ribosome[39]
L-argininamide[40]	Vasopressin[69]	DNA nuclease activity[41]
L-valine[42]	Spectinomycin[70]	Ligase activity[43]
L-isoleucine[44]	L-tyrosinamide[71]	Kinase activity[45]
AMP/ATP[46-50]	HIV-1 RNase H[72]	Phosphoramidate bond cleavage[51]
Guanosine[52]	Chitin[73]	Porphyrin metallation[53]
FMN[47,54]	Human Thrombin[74]	Peroxidase activity[55]
NAD[54]	cAMP[75]	
Vitamin B$_{12}$[56]	Cholic acid[76]	
8-oxo-dG[57]	Hematoporphyrin[77]	
5'-cap[58]	HIV-1 Tat/Zn^{2+}[78]	
	Anthrax spores[79]	

Directed mutagenesis can be used to change one or more properties of a nucleic acid enzyme or its substrate. Using 17E and 17DS as an example, one may wish to alter the avidity of the two arms of the hybridized enzyme and substrate. The "arms" are those areas displaying Watson-Crick base-pairing in

Figure 1. To alter avidity, the length of the arms is changed. Increasing the length of the arms increases the number of Watson-Crick base pairings, thus increasing avidity; decreasing the length decreases avidity. Decreasing the avidity of the arms facilitates the removal of substrate from the enzyme, thus
5   allowing for faster enzymatic turnover.

Another method of decreasing avidity includes creating mismatches between the enzyme and the substrate. Alternatively, the G-C content of the arms may be altered. The effect of any directed change should be monitored to ensure that the enzyme retains the desired activity, including ion sensitivity and
10   selectivity. For example, to ensure that the mutated enzyme maintains sensitivity and selectivity for adenosine, one would test to determine if the mutated enzyme remained reactive in the presence of adenosine (sensitivity) and maintained its lower level of activity in the presence of other effectors (selectivity).

15   In vitro *selection of aptamers*

Aptamers and aptazymes that bind a desired effector can be isolated by *in vitro* selection. *In vitro* selection is a technique in which RNA or DNA molecules with certain functions are isolated from a large number of sequence variants through multiple cycles of selection and amplification (Chapman and Szostak
20   1994, Joyce 1994). DNAzymes and RNAzymes with maximized activities or novel catalytic abilities, as well as aptamers, can be obtained using, for example, the technique of systematic evolution of ligands by exponential enrichment (SELEX) (Tuerk and Gold 1990).

In vitro selection is typically initiated with a large collection (pool) of
25   randomized sequences, usually containing 10^{13}-10^{16} sequence variants. Chemical synthesis of a set of degenerated polynucleotides using standard phosphoramidite chemistry can be used to generate such randomized pools. The 3'-phosphoramidite compounds of the four nucleosides (adenosine, cytosine, guanine, thymidine) are premixed and used to synthesize the
30   polynucleotides; randomness is generated by controlling the ratio of the four

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phosphoroamidites. Biases can also be achieved, as well as holding a phosphoramidite constant at a specific position. Other strategies for creating randomized DNA libraries include mutagenic polymerase chain reaction (PCR) and template-directed mutagenesis (Cadwell and Joyce 1992, Cadwell and

5   Joyce 1994, Tsang and Joyce 1996). If *in vitro* selection of RNA molecules is desired, randomized DNA libraries are first converted to an RNA library by *in vitro* transcription.

The randomized libraries are then screened for molecules possessing a desired function, such as binding the targeted effector, and are isolated.

10   Separation may be achieved using affinity column chromatography (using, *e.g.*, the targeted effector), gel electrophoresis, or selective amplification of a tagged reaction intermediate. The selected molecules are amplified, using, for example, PCR for DNA, or isothermal amplification reaction for RNA. These selected, amplified molecules are then mutated (reintroducing diversity) using, for

15   example, mutagenic PCR to attempt to select for molecules with yet higher activity. These three steps, selection, amplification and mutation, are repeated, often with increasing selection stringency, until sequences with the desired activity dominate the pool.

Novel nucleic acid enzymes isolated from random sequences *in vitro* have

20   extended the catalytic repertoire of RNA and DNA (Table 1). Deoxyribozymes catalyze fewer types of reactions compared to ribozymes (Table 2). The catalytic rate (k_{cat}) of most deoxyribozymes is comparable to that of ribozymes catalyzing the same reaction. In certain cases, the catalytic efficiency (k_{cat}/K_m) of nucleic acid enzymes even exceeds protein enzyme catalytic efficiency. (Santoro and

25   Joyce 1997).

In vitro selection can be used to change the ion specificity or binding affinity of existing nucleic acid enzymes, or to obtain nucleic acid enzymes specific for desired substrates. For example, the Mg^{2+} concentration required for optimal hammerhead ribozyme activity has been lowered using *in vitro* selection

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to improve the enzyme performance under physiological conditions (Conaty et al. 1999, Zillmann et al. 1997).

Often nucleic acid enzymes developed for a specific co-factor by *in vitro* selection will have activity in the presence of other molecules. For example, 17E

5   deoxyribozyme was developed by *in vitro* selection for activity in the presence of Zn^{2+}. However, the enzyme showed greater activity in the presence of Pb^{2+} than Zn^{2+}. Although produced in a process looking for Zn^{2+}-related activity, 17E may be used as a sensitive and selective sensor for Pb^{2+}. To produce nucleic acid enzymes with greater selectivity, a negative selection step may be introduced.

10 (Peter J. Bruesehoff, Jing Li, Anthony J. Augustine III, and Yi Lu, "Improving Metal Ion Specificity During *In Vitro* Selection of Catalytic DNA" *Combinatorial Chemistry and High Throughput Screening* 5, 327-355 (2002)).

Other polynucleotide sequences are useful, including those described in U.S Patent Application Serial Number 09/605,558, filed June 27th, 2000, the

15 contents of which are incorporated by reference (Lu and Li).

Adenosine sensor

The aptazyme shown in Figure 2A is specific for adenosine. The catalytic core of the aptazyme is adapted from the "8-17" DNAzyme and has been

20 optimized for high activity in the presence of Pb^{2+}. The 3´-end of the DNAzyme is linked to the adenosine aptamer motif. Therefore, the presence of adenosine can promote formation of the active tertiary DNAzyme structure. In an aptazyme-based sensor, this complex promotes cleavage of the substrate strand at the single ribo-adenosine position. Without adenosine, even though the three

25 components may still be able to come together by Watson-Crick base-paring, the cleavage activity is dramatically reduced. Also shown are two DNA-functionalized 13 nm diameter gold particles, hybridized to both ends of the aptazyme.

As illustrated in Figure 2B, The substrate strands (both free substrate and substrate hybridized with the enzyme and regulator strands) can act as

30 linkers for the DNA-functionalized gold particles to form aggregates, which

18

have a blue color (reaction A). In the presence of adenosine and metal ions, the substrate can be cleaved (reaction B). The cleaved substrate can no longer act as linkers for particles and the color remains red (reaction C).

In this type of sensor, the presence of the substrate, enzyme and regulator strands is insufficient for aggregate formation at room temperature. It is difficult for all the three strands to hybridize and act as linkers without heating. To solve this problem, another DNA strand ("Ade-linker") is introduced (Figure 8). The purpose of Ade-linker is to hybridize to the substrate strand with high efficiency and facilitate the aggregation of particles at room temperature. After hybridization, the Ade-linker forms a bulged secondary structure with 10 nucleotides. The bulge prevents the Ade-linker from using cleaved substrate as linkers to assemble particles. With the bulge, even if some Ade-linkers hybridize to the cleaved substrate, the whole structure is still floppy, and incapable of assembling particles.

If no adenosine is present, the substrate of the aptazyme hybridizes with Ade-linker, and the hybridized product can assemble particles to form blue aggregates (reaction A). In the presence of adenosine, the substrate strand is cleaved (reaction B), and the cleaved substrate cannot assemble gold particles, yielding red-colored, separated particles (reaction C).

Particles tagged with polynucleotides complementary to the 3' and 5' termini of the nucleic acid enzyme substrate

For the sensor to register enzymatic activity, a detectable change must occur upon a change in aggregation of the particles to which the polynucleotides are attached. In addition, the composition of the particles must be such that they do not interfere with substrate cleavage. Particles may be made of, for example, metals, semiconductors and magnetic materials; ZnS, ZnO, TiO_2, AgI, AgBr, HgI_2, PbS, PbSe, ZnTe, CdTe, $In_2 S_3$, $In_2 Se_3$, $Cd_3 P_2$, $Cd_3 As_2$, InAs, and GaAs (e.g., (Mirkin et al. 2002)); and preferably gold particles (commercially available; e.g., Amersham Biosciences; Piscataway, NJ and Nanoprobes, Inc; Yaphank,

NY). Non-metal particles may also be used, including ceramics and polymers, such as polystyrene latex particles or latex particles containing dye. Preferably the particles have an average particle diameter of at least 20 nm, more preferably at least 30 nm, even more preferably at least 35 nm, including 15-500

5   nm, 20-200 nm, and 35-100 nm.

Gold colloidal particles with a diameter of at least 35 nm are preferred. Gold colloidal particles have high extinction coefficients for the bands that give rise to their intense colors. These colors vary with particle size, concentration, inter-particle distance, extent of aggregation and shape of the aggregates. For

10   instance, the aggregation of gold particles driven by the hybridization of polynucleotides attached to the particles results in an immediate color change visible to the naked eye (see, e.g., (Mirkin et al. 2002)).

The particles, polynucleotides or both are derivatized for the attachment. For instance, polynucleotides derivatized with alkanethiols at their 3'- or 5'-termini

15   readily attach to gold particles (Whitesides 1995). A method of attaching 3' thiol DNA to flat gold surfaces can also be used to attach polynucleotides to particles (Mucic et al. 1996). Alkanethiol-derivatized particles can be used to attach polynucleotides. Other functional groups for attaching polynucleotides to solid surfaces include phosphorothioates, which attach polynucleotides to gold

20   surfaces (Beebe and Rabke-Clemmer 1995), substituted alkylsiloxanes for binding polynucleotides to silica and glass surfaces, aminoalkylsiloxanes and mercaptoaklylsiloxanes (Grabar et al. 1995). Polynucleotides terminating in a 5'-thionucleoside or a 3'-thionucleoside may also be used for attaching polynucleotides to solid surfaces. Some methods of attaching polynucleotides

25   are presented in Table 4.

30

Table 4   Systems for attaching polynucleotides to particles

System	R ference
biotin-streptavidin	(Shaiu et al. 1993)
carboxylic acids on aluminum	(Allara and Nuzzo 1985)
disulfides on gold	(Nuzzo et al. 1987)
carboxylic acids on silica	(Iler 1979, Tompkins and Allara 1974)
carboxylic acids on platinum	(Timmons and Zisman 1965)
aromatic ring compounds on platinum	(Soriaga and Hubbard 1982)
silanes on silica	(Maoz and Sagiv 1987)

Particle alignment

In order for the particles to align in the "tail-to-tail" manner, the particles
5   are functionalized in the following fashion. Particles that bind to the substrate at
a site 5' of the substrate are functionalized with complementary polynucleotide
strands that are linked at their 3' termini to the particles, whereas particles that
bind to the substrate at a site 3' of the substrate are functionalized with
complementary polynucleotide strands that are linked at their 5' termini to the
10   particles. The substrate-particle complex has the structure illustrated in Figure
3B, rather than the structure illustrated in Figure 3A.

Preferably, the substrate is modified to facilitate annealing to the
complementary polynucleotide strand attached to the particles, for example, by
extension of the 3'- and 5'- ends by a number of bases that act as "sticky ends".
15   Substrate modification allows complexes comprising substrate-linked particles to
be formed without inhibiting the nucleic acid enzyme/substrate interaction.
However, where the substrate contains regions not critical for interaction with the
nucleic acid enzyme, modification may not be necessary.

It is also possible for the particles to be attached directly to the substrate,
20   or alternatively, for one end of the substrate to be attached to a particle, and for
the other end to be complementary to a polynucleotide which is attached a
particle. Therefore, the complementary polynucleotides are optional.

21

To detect the target cofactor or effector, the nucleic acid enzyme, substrate, and labeled particles are combined in the presence of a sample suspected of containing a target cofactor or effector, such as adenosine, to which the enzyme is sensitive (Figure 3). In the presence of the cofactor or effector,

5   the enzyme cleaves the substrate, preventing aggregate formation.

Different aggregation states of the particles results in different colors. For example, a large degree of gold particle aggregation displays blue colors while a small degree of particle aggregation displays red colors. Furthermore, the amount of substrate cleavage and thus the degree of aggregation depends on

10   the concentration of the cofactor or effector. A low cofactor or effector concentration results in only partial substrate cleavage that produces a mixture of single particles and aggregates, allowing for semi-quantitative or qualitative assays. The color difference can be amplified to improve sensitivity. For a quantitative measurement, the optical spectra of the assay mixture are

15   determined. In addition to color change, the formation of aggregates of the particles, or precipitation of aggregated particles may also be monitored. Color changes can be observed with the naked eye or spectroscopically. The formation of aggregates can be observed by electron microscopy or by nephelometry; precipitation of aggregated particles can be observed with the

20   naked eye or microscopically.

To facilitate the observation of a color change, the color may be observed on a background of a contrasting color. When gold particles are used, the observation of a color change is facilitated by spotting a sample of the hybridization solution on a solid white surface (such as silica or alumina TLC

25   plates, filter paper, cellulose nitrate membranes, and nylon membranes) and allowing the spot to dry. Initially, the spot retains the color of the hybridization solution (which ranges from pink/red, in the absence of aggregation, to purplish-red/purple, if there has been aggregation of gold particles). On drying, a blue spot develops if aggregation is present prior to spotting; a pink spot develops if

30   dispersion occurred. The blue and the pink spots are stable and do not change

22

on subsequent cooling or heating or over time. They provide a convenient permanent record of the test. No other steps are necessary to observe the color change.

Alternatively, assay results may be visualized by spotting a sample onto a

5   glass fiber filter for use with 35 nm gold particles. After rinsing with water, a spot comprising the aggregates is observed. Additional methods are also available for visualizing assay results (Mirkin et al. 2002).

The targeted cofactor or effector can be detected in a variety of samples, including biological samples. Standards containing known amounts of the

10   cofactor or effector may be assayed along side the unknown sample, and the color changes compared. Alternatively, standard color charts, similar to those used with pH papers, may be provided.

Kits

The invention also provides kits for detecting analytes as cofactors or

15   effectors. In one embodiment, the kit includes at least one container, the container holding at least two types of particles having polynucleotides attached thereto; a substrate, the substrate having at least three portions, the first portion being 5' to the second portion, the second portion being cleaved by the nucleic acid enzyme in the presence of the analyte, and the third portion being 3' to the

20   second portion; and a nucleic acid enzyme. The polynucleotides attached to the first particles have a sequence complementary to the sequence of at least the first portion of the substrate and are attached to the particles at their 5' termini. The polynucleotides attached to the second particles have a sequence complementary to the sequence of at least the third portion of the substrate, and

25   are attached to the particles at their 3' termini.

When a kit is supplied, the different components of the composition may be packaged in separate containers and admixed immediately before use. Such packaging of the components separately permits long-term storage of the active components. For example, one of more of the particles having polynucleotides

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attached thereto; the substrate; and the nucleic acid enzyme are supplied in separate containers.

The reagents included in the kits can be supplied in containers of any sort such that the life of the different components are preserved and are not adsorbed

5   or altered by the materials of the container. For example, sealed glass ampules may contain one of more of the reagents, or buffers that have been packaged under a neutral, non-reacting gas, such as nitrogen. Ampules may consist of any suitable material, such as glass, organic polymers, such as polycarbonate, polystyrene, *etc.*; ceramic, metal or any other material typically employed to hold

10   similar reagents. Other examples of suitable containers include simple bottles that may be fabricated from similar substances as ampules; and envelopes that may comprise foil-lined interiors, such as aluminum or an alloy. Other containers include test tubes, vials, flasks, bottles, syringes, or the like. Containers may have a sterile access port, such as a bottle having a stopper that can be pierced

15   by a hypodermic injection needle. Other containers may have two compartments that are separated by a readily removable membrane that upon removal permits the components to be mixed. Removable membranes may be glass, plastic, rubber, *etc.*

The kits may also contain other reagents and items useful for detecting

20   the targeted cofactor or effector. The reagents may include standard solutions containing known quantities of the cofactor or effector, dilution and other buffers, pretreatment reagents, *etc..* Other items which may be provided as part include a backing (for visualizing aggregate break down), such as a TLC silica plate; microporous materials, syringes, pipettes, cuvettes and containers. Standard

25   charts indicating the appearance of the particles in various aggregation states, corresponding to the presence of different amounts of the cofactor or effector being tested, may be provided.

Kits may also be supplied with instructional materials. Instructions may be printed on paper or other substrate, and/or may be supplied as an electronic-

30   readable medium, such as a floppy disc, CD-ROM, DVD-ROM, Zip disc,

videotape, audiotape, *etc.* Detailed instructions may not be physically associated with the kit; instead, a user may be directed to an internet web site specified by the manufacturer or distributor of the kit, or supplied as electronic mail.

5 EXAMPLES

Example 1   Colorimetric Pb^{2+} biosensor

<u>Polynucleotides and reagents</u>

All polynucleotides were purchased from Integrated DNA Technology Inc. (Coralville, IA). Adenosine and other nucleosides were purchased from Sigma-

10 Aldrich (St. Louis, MO). Thirty-five nm diameter gold particles were prepared and 3'- and 5'-thiol-modified 12-mer DNA was attached (Storhoff et al. 1998). The substrates and the enzymes were purified by HPLC.

<u>Preparation and functionalization of gold particles</u>

15 Thirty-five nm diameter gold particles were prepared by the reduction of $HAuCl_4$ by sodium citrate. Glassware used in the preparation was soaked in aqua regia and rinsed thoroughly with Millipore water. In a 250 mL two-neck flask, 200 mL 0.3 mM $HAuCl_4$ was heated to reflux under stirring. Two mL 38.8 mM sodium citrate was then added. Within several minutes, the color changed

20 from pale yellow to deep red. After the color change, the system was allowed to reflux for another half hour for reduction to complete. Then the system was cooled slowly to room temperature, and the particles were filtered with a Pyrex funnel. The particles were characterized using transmission electron microscopy (TEM) on a JEOL 2010 electron microscope, and the size was determined to be

25 35 ± 6 nm. Most of the particles had a spherical shape, and a small portion of particles were rods with aspect ratios less than two. The gold colloid had an extinction peak at 532 nm of ~1, and the concentration was calculated to be approximately 0.23 nM by assuming all particles were spherical with radii of 35 nm; all $HAuCl_4$ was reacted and the density of particles were the same as bulk

30 gold. This gave an extinction coefficient of 4.4×10^9 M^{-1} cm^{-1} at 532 nm.

25

<u>Functionalization of particles with 3'- and 5'-thiol modified DNA</u>

Thiol-modified DNA was activated by incubating with 50 mM dithiotreitol (DTT). Typically, 60 μL of 1 mM DNA were incubated with 60 μL 100 mM DTT at

5   room temperature (20-22 °C) for 2 hours. The mixture was then placed onto a Sep-Pak C18 column to remove DTT. The column was treated with 10 mL 95% CH_3CN, 10 mL mixture of CH_3CN, methanol and water with 1:1:1 volume ratio, 20 mL water and 10 mL 2 M NH_4OAc. DNA and DTT mixture were then loaded into the column. DTT was washed away by 20 mL de-ionized water, and DNA

10   was eluted by 1 mL of 95% CH_3CN, 10 mL mixture of CH_3CN, methanol and water with 1:1:1 volume. The eluted DNA was added to 12 mL of gold particles (0.23 nM). After incubation for one day, 1.4 mL of buffer containing 1 M NaCl and 0.1 M Tris-acetate (TA), pH 7.2 was added. After another incubation of 2 days, the particles were collected by centrifugation at 8000 rpm for 10 minutes.

15   Supernatants were removed, and the DNA-functionalized particles were redispersed in 25 mM TA buffer, pH 7.2, 100 mM NaCl. The process was repeated three times to ensure that free DNA was removed; and finally, the particles were dispersed in the same buffer and the extinction coefficient at 532 nm was adjusted to ~2.2 (corresponding to a concentration of ~0.5 nM).

20

<u>Aggregation of particles by the DNAzyme</u>

In a typical experiment, 20 μL of 3'-thiol modified and 20 μL of 5'-thiol modified DNA functionalized gold particles, both at a concentration of ~0.5 nM, were mixed. NaCl, the enzyme (17E) and Pb^{2+} were added, and the final volume

25   was adjusted to 100 μL upon addition of the substrate. The aggregation was initiated by adding the substrate strand, and the extinction was monitored on a Hewlett-Packard 8453 spectrophotometer. The buffer was pH 7.2 TA for all experiments, except for pH-dependent studies. The time between substrate addition to the monitoring of the first spectrum was controlled to be 15 seconds

30   for all samples.

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In the experiments illustrated in Figure 4, the extinction ratio was normalized for better comparison. These experiments were run with 2 μM enzyme, the substrate concentration was 160 nM for 13 nm particles and 120 nM for 13 nm-35 nm mixed particles and 3 nM for 35 nm-35 nm particles. The buffer was pH 7.2, 25 mM TA.

Optimization of the new Pb^{2+} sensor system

(f) The extinction ratio after 10 minutes of aggregation.

Clear red to blue color changes can be observed upon aggregation of the 35 nm gold particles, thus the aggregation process can be conveniently monitored by UV-visible spectroscopy. Shown in Figure 6A are several spectra of the new sensor system with 3 nM of the linking substrate, NaCl 250 mM, and enzyme (17E) 1 μM, at different time points after the addition of the substrate strand. Before the substrate strand was added, the system showed a red color with an extinction peak at 532 nm, which was 10 nm red shifted compared to 13 nm diameter particles. In the first 3 minutes, the color change was very small. During this period it was thought that the substrate strands were base pairing with the enzyme strand, and the hybridized DNAzymes were linking to gold particles. In the next several minutes, the rate of color change was very fast, suggesting that the particles were cross-linking to form large aggregates. With the formation of aggregates, the extinction at the 532 nm peak decreased, while it increased in the 700 nm region, yielding the red-to-blue color transition. After 10 minutes, the aggregates began to precipitate, resulting in the decrease of observed extinction at all wavelengths. To minimize the effects of differences in sample preparations and concentration of particles, the extinction ratio of 532 nm and 700 nm was used to monitor the rate of color change, with a higher ratio associated with red color, and a lower ratio associated with blue color.

Illustrated in Figure 6B are the rates of color change in the presence of different substrate concentrations. Higher substrate concentrations yielded higher rates of color change. Thus, the decrease of substrate concentration resulted in a

27

"less blue" or a "more red" color at a set time. Different Pb^{2+} concentrations cleaved the substrate at different rates; thus, by inspecting the color displayed by the sensor, Pb^{2+} was detected and quantified. The experiments were performed using 3 nM substrate, NaCl 250 mM and 17E 1 μM.

5

Additional factors influencing aggregation.

As outlined above, other factors were found to affect the rate of aggregation of particles, such as the enzyme concentration, salt concentration and pH values. The experiments illustrated in Figure 6C were all run with 3nM

10   substrate concentration and 1 μM 17E enzyme concentration, and with varying NaCl concentrations. Higher NaCl concentrations favored aggregation, likely because NaCl reduced the electrostatic repulsion between the negative charges of the negatively charged DNA.

The enzyme concentration also had significant effect on the aggregation

15   of particles. The experiments illustrated in Figure 6D were all run at 0.3 nM substrate concentration and 250 mM NaCl concentration, and with varying 17E enzyme concentrations. Higher concentrations of the enzyme strand yielded faster aggregation rates. In the absence of the enzyme or when the enzyme concentration was very low (Figure 6D, topmost curve), aggregation was

20   inhibited. Although physically only the substrate strand was acting as linkers to assemble particles, the presence of the enzyme strand appears to be optional. From these observations, it was hypothesized that the first step of aggregation is the hybridization of the substrate strand to the enzyme strand. Then the hybridized DNAzyme can assemble gold particles. In the absence of the enzyme

25   strand, the substrate by itself is likely to be too floppy to assemble gold particles.

Different pH values also influenced aggregation. As shown in Figure 6E, from pH 5.2 to pH 10.2 (pH 5.2, acetate buffer, pH 6.2 MES buffer, pH 7.2-10.2, TA buffer), all the samples gave a sigmoidal curve, which was characteristic for the DNAzyme directed assembly of gold particles at room

30   temperature (20-22 °C). For the pH 5.2 sample, the aggregation of particles

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appeared inhibited. The extinction ratio after 10 minutes aggregation was plotted and is presented in Figure 6F. From pH 7.2 to 9.2, similar extinction properties have been observed, suggesting a similar rate of aggregation, while the rate of aggregation decreases for both higher and lower pH. The sensor may thus be

5   used in the pH range from about 6.2 to about 10.2, with optimal performance from about pH 7.2 to about 9.2. The change of rate of particle aggregation was likely due to protonation and deprotonation of the DNA bases at low or high pH, which affected the base pairing, thus leading to a change in aggregation rate.

10   <u>Aggregation at room temperature</u>

The kinetics of aggregation at room temperature (20-22 °C) of the new sensor with different Pb^{2+} concentrations was monitored by UV-visible spectroscopy. It is hypothesized that in the presence of Pb^{2+}, after the hybridization of the substrate to the enzyme strand, the substrate strand could be

15   either cleaved by the enzyme strand or be a linker to assemble particles. The relative rate of the two processes depends on the concentration of Pb^{2+}. As shown in Figure 7A, as Pb^{2+} concentration increased, the rate of aggregation was suppressed. For the sample without Pb^{2+} added, the aggregation was almost complete after 10 minutes. The extinction ratio at 10 minutes was used to

20   quantify Pb^{2+} concentration (Figure 7B).

Example 2   Colorimetric adenosine biosensor

<u>Aggregation of particles by the aptazyme</u>

To determine the optimal concentration of the substrate strand for the

25   aptazyme-based sensor, 20 μL of each particle solution (E_{532} = 2.2) were mixed and Ade-linker was added to a final concentration of 5 μM, NaCl to a final concentration of 250 mM, and the volume was adjusted to 100 μL after adding the substrate. The solution was in 25 mM TA buffer, at pH 7.2.

The solution was used to prepare a second solution with a final

30   concentration of 0.15 μM substrate strand, 3 μM enzyme and regulator strand, 5

<center>29</center>

mM adenosine, 250 mM NaCl and 25 mM TA buffer, pH 7.2. Ninety-eight μL of the second solution were transferred in a reaction tube, and this moment was set as the 0 point. Two μL of metal ions stock solution was added to the reaction tube to initiate the cleavage reaction, and 2 μL aliquots were transferred into

5   detection tubes at different time points. Once each aliquot was transferred from the reaction tube to a detection tube, the cleavage reaction was quenched by the addition of EDTA in the detection tube. A 2 μL aliquot from the reaction tube contained 0.3 pmol substrate and would have given a substrate concentration of 3 nM in the detection tube if no cleavage occurred. The extinction spectrum of .

10   each sample in the reaction tube was monitored at 20 minutes after the transfer of the aliquot from the reaction tube.

To test of the sensitivity and selectivity of the sensor, different reaction tubes were prepared with different concentrations of adenosine or other nucleosides. After 30 minutes of incubation, 2 μL aliquots was transferred to

15   detection tubes, and the extinction spectra of the detection tubes were measured after 20 minutes of incubation.

Figure 9A illustrates the substrate concentration-dependent aggregation of particles, measured by the extinction spectra after 20 minutes of aggregation. Figure 9B illustrates the kinetics of the cleavage of the

20   substrate by the aptazyme. Figure 9C shows the sensitivity and selectivity of the newly designed aptazyme-based sensor for adenosine. The *pink, red and blue* dots are the extinction ratio of 5 mM of cytidine, gaunosine and uridine, respectively.

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